Woon JSK, King PJH, Mackeen MM, Mahadi NM, Seman WMKW, Broughton WJ, Murad AMA, Bakar FDA. Cloning, Production and Characterization of a Glycoside Hydrolase Family 7 Enzyme from the Gut Microbiota of the Termite *Coptotermes curvignathus*. *Molecular Biotechnology* 2017, 59(7), 271-283.

Cloning, Production and Characterization of a Glycoside Hydrolase Family 7 Enzyme from the Gut Microbiota of the Termite *Coptotermes curvignathus*

James Sy-Keen Woon (james.woon@newcastle.edu.my) [1,2], Patricia King Jie Hung (patricia@btu.upm.edu.my) [3], Mukram Mohamed Mackeen (mukram@ukm.edu.my) [4,5], Nor Muhammad Mahadi (nor.mahadi@gmail.com) [5,6], Wan Mohd Khairulikhsan Wan Seman (ikhsan.ws@gmail.com)[6], William J. Broughton (williamebroughton@gmail.com)[7], Abdul Munir Abdul Murad (munir@ukm.edu.my) [1] and Farah Diba Abu Bakar [1*]

[1] School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.

[2] Newcastle University Medicine Malaysia, Faculty of Medical Sciences, 79200 Iskandar Puteri, Johor, Malaysia. (Present address)

[3] Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Bintulu Sarawak Campus, 97008 Bintulu, Sarawak, Malaysia.

[4] School of Chemical Sciences and Food Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.

[5] Institute of Systems Biology (INBIOSIS), Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.

[6] Malaysia Genome Institute, Jalan Bangi Lama, 43000 Kajang, Selangor Malaysia.

[7] Department 4 (Materials & Environment), Federal Institute of Materials Research and Testing, Unter den Eichen 87, Berlin, 12205, Germany.

Corresponding author: Email: fabyff@ukm.edu.my

Running title: Expression of a termite GHF7 enzyme

Abstract: *Coptotermes curvignathus* is a termite that, owing to its ability to digest living trees, serves as a gold mine for robust industrial enzymes. This unique characteristic reflects the presence of very efficient hydrolytic enzyme systems including cellulases. Transcriptomic analyses of the gut of *C. curvignathus* revealed that carbohydrate-active enzymes (CAZy) were encoded by 3,254 transcripts and that included 69 transcripts encoding glycoside hydrolase family 7 (GHF7) enzymes. Since GHF7 enzymes are useful to the biomass conversion industry, a gene encoding for a GHF7 enzyme (*Gh*1254) was synthesized, sub-cloned and expressed in the methylotrophic yeast *Pichia pastoris*. Expressed GH1254 had an apparent molecular mass of 42 kDa but purification was hampered by its low expression levels in shaken flasks. To obtain more of the enzyme, GH1254 was produced in a bioreactor that resulted in a four-fold increase in crude enzyme levels. The purified enzyme was active towards soluble synthetic substrates such as 4-methylumbelliferyl-β-D-cellobioside, 4-nitrophenyl-β-D-cellobioside and 4-nitrophenyl-β-D-lactoside but was non-hydrolytic towards Avicel or carboxymethyl cellulose. GH1254 catalyzed optimally at 35 ^{o}C and maintained 70% of its activity at 25 ^{o}C. This enzyme is thus potentially useful in food industries employing low temperature conditions.

Keywords: Avicel, biomass, bioreactor, cellulase, saccharification, symbiont.

Abbreviations: CMC, carboxymethyl cellulose; MUC, 4-methylumbelliferyl β-D-cellobioside; *p*NPC, *p*-nitrophenol-*β*-D-cellobioside; *p*NPG3, *p*-nitrophenol-*β*-D-cellotrioside; *p*NPL, *p*-nitrophenol-*β*-D-lactoside.

Introduction

Symbionts in the guts of termites permit the decomposition of wood [1-2]. Some are able to convert 95% of cellulose into simple sugars in less than 24 h [3]. Gut symbiont consortia consist of archaea, bacteria and flagellates that have co-evolved with their host. Termite adaptations to different environments are reflected in the composition of gut microbiota that provide optimized hydrolytic activities for nutrient release as well as protection against opportunistic pathogens [4]. Brune called the termite gut 'the world smallest bioreactor' [5-6]. Thus, understanding lignocellulolytic interactions in termite guts may provide insights and potential novel enzymes useful in the biomass conversion industry [7].

Termites are classified into higher- and lower-termites based on the presence or absence of flagellated protistan symbionts in their hindguts. Only one of the seven termite families (Termitidae) belongs to the higher group lacking flagellates. Other families (Hodotermitidae, Kalotermitidae, Mastotermitidae, Rhinotermitidae, Serritermitidae and Termopsidae – collectively the lower termites) contain flagellates in their hindguts and secrete β-glucosidases (EC 3.2.1.21) or endo-β-1,4-glucanases (EC 3.2.1.4) through their salivary glands [8]. Together with the physical grinding action of the muscular gizzard, these enzymes partially digest wood particles in the foregut. Glucose released in the digestion process is absorbed in the mid-gut while the remaining digested wood particles are passed into the voluminous hindgut where they are phagocytized by flagellates and hydrolyzed by powerful cellulases and

hemicellulases in digestive vacuoles. Hindgut bacteria assume the role of the flagellates in cellulose degradation in higher termites [6].

Coptoptermes curvignathus also known as the rubber termite is the largest species of *Coptotermes* spp. in Asia. It is whitish due to the storage of white latex used in defence [9]. Unlike most other termite species that feed on partially degraded wood, *C. curvignathus* is capable of attacking and consuming tissues of healthy living trees. This unique digestive feature shows that *C. curvignathus* can overcome plant defences and perform efficient conversion of lignocellulose into simpler compounds [4].

Cellulases are enzymes that belong to the glycosyl hydrolase family (GHF) hydrolysing β-1,4-glycosydic bonds between glucose subunits in cellulose polymers [10]. To breakdown cellulose efficiently, three major categories of cellulases are often needed to work in synergy, i.e. cellobiohydrolases (CBHs, EC 3.2.1.91), endoglucanases (EGLs, EC 3.2.1.4) and β-glucosidases (BGLs, EC 3.2.1.21) [11]. CBHs are exo-acting enzymes that cleave from the opposite ends of cellulose chains [12-15] and are only found in the hindgut of lower termites [7, 14, 15]. EGLs are endo-acting enzymes that initiate random cuts in the central region of cellulose chains. BGLs are cellobiases that cleave cellobioses into glucose subunits [12]. Both EGLs and BGLs are found in the midgut and hindgut of lower termites [6]. Using a baculovirus-insect expression system, Sethi *et al.* [16] engineered a termite (*Reticulitermes flavipes*) cellulase possessing multiple activities including CBH, EGL and BGL.

Nevertheless, due to lower expression levels and specific activities, expression of recombinant cellobiohydrolases has been challenging (17, 18). To increase both expression levels and specific activities, we synthesized, sub-cloned and expressed the gene encoding a putative cellobiohydrolase (*Gh*1254) from *C. curvignathus* in the

methylotrophic yeast *P. pastoris* which strongly over-expresses foreign proteins [19] and can serve as an expression system for insect proteins [20].

Materials and Methods

Microbial strains and cloning vectors

Escherichia coli strain DH5α (Promega, Madison, WI, USA) was used for plasmid manipulation and propagation as described by Sambrook and Russell [21]. *Pichia pastoris* strain X-33 (Invitrogen/Life Technologies, Grand Island, NY, USA) was used for the expression of *C. curvignathus Gh*1254. Preparation of media and growth of the yeast cells were performed following Invitrogen/Life Technologies *Pichia* expression system protocols.

Screening for cellobiohydrolase-encoding genes in the gut transcriptome

Transcriptomic data were generated from RNA extracted from the digestive tracts of 200 worker termites collected in Bintulu, Sarawak, Malaysia. Before use the termites were kept in their own nest wood, in a plastic container in the dark. Then, salivary glands, foreguts, mid-guts, and hindguts were dissected from the termites and frozen in liquid nitrogen. Total RNA was extracted with Sepasol RNA 1 Super G (Nacalai Tesque Inc, Nijo Karasuma, Nakagyo-ku, Kyoto, 604-0855, Japan) according to the protocols of the manufacturer. cDNA libraries were constructed using the TruSeq RNA kit (Illumina, San Diego, CA, USA) and sequenced using the Illumina HiSeq 2000 platform. The raw sequence data was pre-processed (DynamicTrim) using reads a minimum sequence length of 50 bp (LengthSort) and a Phred value at QV > 20. Consensus sequences were assembled using Oases [22]. The

combined consensus sequence data was annotated using BLAST similarity searches against the NCBI database and BLASTX [23] (cut-off value 1e-5). Functional categories were assigned using gene ontology (GO) databases according to the top hits in the BLASTX searches.

Afterwards, the transcript sequences were translated into amino acid sequences using the ExPASy Translate Tool (http://web.expasy.org/translate/). To predict the functions of the translated sequences, protein domain analyses were performed using the InterProScan 5 server (http://www.ebi.ac.uk/Tools/pfa/iprscan5/). Sequences that contained family 7 glycosyl hydrolase (GHF7) domains were selected for further analyses. There are at least four classes of enzymes grouped under GHF7, i.e. cellobiohydrolases, endo-1,3-β-glucanases, endo-1,4-β-glucanases and chitosanases. [24] To identify the putative function of GH1254, selected transcripts were analysed using the BLASTX server (http://blast.ncbi.nlm.nih.gov/). Out of top 100 hits, nearly all hits are annotated as GHF7 proteins. Therefore, the putative function of GH1254 is unclear based on bioinformatics analyses alone.

A phylogenetic tree of termite and fungal cellulases was constructed using Molecular Evolutionary Genetic Analysis (MEGA6) software [25]. Three GHF7 sequences (KC751534, KC751535 and KC751536) from the termite *R. flavipes* [16] along with others well characterized fungal CBHs were included. The cellulase of *Ruminococcus* sp. (GenBank ID: CDE11952) was used as an outgroup to build the phylogenetic tree.

Synthesis of *Gh*1254

Codon usage of *Gh*1254 was optimized for expression in *P. pastoris* and synthesized in an Invitrogen/Life Technologies cloning vector. *Kpn*I and *Xba*I

restriction adaptors were added to the 5'- and 3'- ends of the gene to facilitate sub-cloning of *Gh*1254 into the expression vector, pPICZαC (Invitrogen/Life Technologies). *Gh*1254 was inserted into the multiple cloning site of pPICZαC downstream of the alpha-factor signal peptide. Six histidine-encoding codons (CAT) followed by a stop codon (TGA) were added to the 3'- end of *Gh*1254.

Transformation of *P. pastoris* X-33

Transformation of *P. pastoris* X-33 was performed according to the instruction manual of Invitrogen/Life Technologies using an electroporator (Model 2510, Eppendorf, Hamburg, Germany) at 1500 V. Positive transformants were confirmed by PCR [26] using a pair of *AOX*1 primers targeting the insert flanked by the *AOX*1 sequences. Transformants were selected on YPDS (yeast extract/peptone/dextrose medium plus sorbitol) plates containing Zeocin^{TM} (2,000 μg.mL^{-1}).

Production of GH1254

Transformants carrying the expression cassette pPICZαC_Gh1254 were inoculated in 400 mL of buffered glycerol complex medium (BMGY) and cultured to an OD_{600} of 2 - 3 at 30 °C, 240 rpm. Then, yeast cells were harvested via centrifugation (1,500 g, 5 min) and re-suspended in 200 mL of buffered methanol complex medium (BMMY). Methanol was added to a final concentration of 1.0% (v/v) during 24 h incubation (at 30 °C, 240 rpm). Culture supernatants were clarified by centrifugation (3,000 g, 5 min) and stored at −20 °C.

To increase production of GH1254, fermentation was performed in a 7.5 L Labfors bioreactor (working volume – 3 L) (Infors AG, Bottmingen, Switzerland). A fresh transformant colony was first cultured in 10 mL yeast peptone dextrose (YPD) broth

overnight at 30 °C then, this culture was inoculated into 290 mL of YPD broth and incubated for 36 h at the same temperature. Finally, the culture was transferred into the bioreactor containing 2.7 L Basal Salts Medium (BSM) and 16.1 mL of Pichia Trace Metal 1 solution [27]. The fermenter was operated in batch mode at 28 °C until the glycerol was depleted as indicated by a spike in dissolved oxygen concentration (after 36 h). To further increase cell densities, fed-batch conditions using a glycerol feed [50% (v/v) glycerol + 7.2 mL PTM1 per litre glycerol] with a minimum feed rate of 0.125 $mL.min^{-1}$ and a maximum feed rate of 0.25 $mL.min^{-1}$ for 11 h. A starvation period of 1 h was then imposed, followed by methanol induction with the same feed rate (100% methanol + 7.2 mL PTM1 per litre methanol) at 25 °C for 40 h. The antifoaming agent J673A Struktol (Schill + Seilacher Struktol GmbH, Hamburg, Germany) was used at 5% (v/v) to control foaming. The temperature was maintained at 25 °C and agitation was set to 600 rpm but cascaded between 200 and 1000 rpm. When necessary, pure oxygen was supplied through gas mixing to maintain the partial-pressure of oxygen at 40%. The aeration rate was held at 2.5 vvm. The production of GH1254 was verified by SDS-PAGE (12% polyacrylamide) followed by western-blot analyses using mouse anti His-tag monoclonal antibodies (Novagen, Madison, USA) and a HRP-conjugated anti-mouse antibodies (Promega) for chemi-luminescent detection on X-ray films. Protein concentrations were determined using the Bradford method [28]. Samples were taken at 4 to 12 h intervals to detect for enzymatic activities along with yeast cell densities (OD_{600}) and dry-cell weights.

Purification of GH1254

Culture supernatants containing His-tagged GH1254 were purified by Immobilized Metal-ion Affinity Chromatography (IMAC) using a 1 mL HiTrap chelating column

charged with Ni^{2+} (AKTA Prime system - GE Healthcare Bio-Sciences Corp., NJ, USA). The column was equilibrated with 10 mL binding buffer containing 20 mM of imidazole. 1 mL of concentrated enzyme was loaded and eluted with a 50-400 mM imidazole gradient. Fractions that contained high concentrations of proteins were pooled, diluted two-fold with 50 mM sodium citrate buffer (pH 4) and concentrated using Vivaspin centrifugal concentrators (cut-off - 10 kDa - GE Healthcare Bio-Sciences Corp.).

Enzyme assays

The catalytic activity (initial rates) of GH1254 was measured using the fluorogenic substrate, 4-methylumbelliferyl-β-D-cellobioside (MUC) (Sigma–Aldrich Corp., St. Louis, MO) in a 400 μL reaction mixture containing 30 mM citrate buffer (pH 4.0), 0.5 mM MUC and 3 μg of purified GH1254 incubated for 15-180 min at 15 min intervals. Subsequently, enzyme assays were incubated at 35 °C for 20 min (within the linear enzyme activity phase) and the reactions terminated by adding 100 μL of 1.0 M Na_2CO_3. Fluorescence of the hydrolyzed product (methylumbelliferone) was determined at excitation and emission wavelengths of 365 and 460 nm, respectively.

Enzyme assays were also performed under similar conditions using chromogenic substrates including *p*-nitrophenol-β-D-cellobioside (*p*NPC), *p*-nitrophenol-β-D-cellotrioside (*p*NPG3) and *p*-nitrophenol-β-D-lactoside (*p*NPL) (Sigma-Aldrich), at a final concentration of 0.5 mM. To detect nitrophenol the hydrolysis product, optical densities were read at 405 nm.

Enzyme activity of GH1254 was assayed against the insoluble microcrystalline substrate, Avicel® PH-101 (Sigma–Aldrich) and Sigmacell® cellulose as well as the soluble substrate, carboxymethyl cellulose (CMC) both at a final concentration of 1%

(w/v) using 3 μg of purified GH1254. Incubation was at 35 °C for 60 min and boiling (10 min) used to terminate the reactions. Amounts of reducing sugar produced were estimated using the dinitrosalicylic (DNS) reagent method of Miller [29].

The optimum temperature and pH of GH1254 were determined by incubating the reaction mixtures at 25 to 50 °C and pH (3 to 10) using 0.5 mM MUC (final concentration) as the substrate for 20 min. One unit of enzyme activity was defined as the amount of product (4-methylumbelliferrone, *p*NP or reducing sugar) in μmol produced per minute under standard condition.

All enzyme assay experiments were repeated at least twice in triplicates.

Thermal and pH stability of GH1254

Purified GH1254 (3 μg) was incubated at various temperatures (25, 30, 35, 40, 45 and 50 °C) in 250 μL citrate buffer (pH 4.0, 30 mM) for 30 min. Upon cooling down on ice for 1 min, MUC (at 0.5 mM final concentration) and deionized distilled water were added to top up the reaction mixtures to a final volume of 400 μL. Residual activities of the treated protein was assayed using the standard protocol described above and MUC as the substrate.

Purified GH1254 (3 μg) was also incubated at various pH buffers (pH 3–8) for 30 min. Subsequently, MUC (at 0.5 mM final concentration) and deionized distilled water were added to top up the reaction mixtures to a final volume of 400 μL. Residual activities of the treated protein was assayed using the standard protocol described above and MUC as the substrate.

Kinetic constants of GH1254

Michaelis–Menten kinetics of purified GH1254 were determined at 35 °C in 30 mM citrate buffer (pH 4.0) with concentrations of MUC ranging from 0.1 to 2.0 mM. Both K_m and V_{max} constants were calculated based on Lineweaver-Burk plot [30].

Effect of metal ions and reagents

Reaction mixtures comprised 3 μg enzyme, 250 μL 30 mM citrate buffer (pH 4.0), 100 μL 1 mM MUC and 50 μL buffer containing different metal ions - Ba^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+}, and Zn^{2+} at final concentrations of 1 and 10 mM. The effects of SDS (0.1 % and 1.0 % w/v) on enzyme activity under standard conditions were also tested.

Results and Discussion

Cellulases-encoding genes in the gut transcriptome

Transcriptomics data revealed 3,254 carbohydrate-active enzyme (CAZy) related transcripts from the gut of *C. curvignathus* which consisted 69 transcripts encoding GH family 7 enzymes. Transcripts encoding GHF7 and GHF6 proteins were especially targeted in this study since they tend to harbour fungal cellobiohydrolases which are essential in the exo-exo type synergistic degradation of cellulose [12]. GHF7 cellobiohydrolases hydrolyze cellulose from the reducing terminus whilst GH 6 cellobiohydrolases degrade cellulose from the non-reducing end [31]. GH family 6 transcripts were not found in the gut of *C. curvignathus* however, suggesting that this termite might employ different digestive strategies to utilize biomass.

BLASTX analyses revealed that most GHF7 transcripts from the gut of *C. curvignathus* matched predominantly to fungal cellobiohydrolases or unspecified

GHF7 proteins in the top one hundred hits when RefSeq database (https://www.ncbi.nlm.nih.gov/refseq/) was used as reference sequences. Since successful heterologous expression of recombinant genes is dependent on the complexity of post-translational modifications including disulfide bond formation and glycosylation [32], one transcript (HPK2EODRW_1254) was selected due to its relatively low number of disulfide bonds (6 disulfide bonds as predicted by the DiANNA Server, http://clavius.bc.edu/~clotelab/DiANNA/) and the lack of *N*-glycosylation site as predicted by the NetNGlyc Server (http://www.cbs.dtu.dk/services/NetNGlyc/).

Together with KC751534, GH1254 diverges from other cellulases forming a distinct clade within the GHF7 group (Fig.1). KC751534 is a GHF7 cellulase from the termite *R. flavipes*. Sethi et al. (2013) demonstrated that KC751534 exhibited a combination of cellobiohydrolase, endoglucanase and β-glucosidase activities [16]. Two other termite GHF7 cellulases of *R. flavipes* (KC751535, KC751536) also diverge from fungal cellulases, but less profoundly than GH1254. This uniqueness of GH1254 might reflect different biomass conversion strategies.

A multiple sequence alignment of GH1254 from *C. curvignathus* along with other cellulases (*R. flavipes* KC751534, *T. reesei* CBHI and EGI [33]) showed that these glycoside hydrolases share similar secondary structures but both GH1254 and KC751534 lack a type 1 cellulose binding module (CBM1) that facilitates binding of crystalline cellulose [13] (Fig. 2).

(FIGURE 1)(FIGURE 2)

Introduction of *Gh*1254 into *P. pastoris*

297 Transformation of *P. pastoris* with the expression cassette (pPICZαC_Gh1254)
298 yielded > 30 transformants, 16 of which were randomly picked and re-streaked on
299 YPDS plates containing 500 μgmL^{-1} Zeocin^{TM}. All tranformants grew well but upon
300 transfer to 1,000 μg.mL^{-1} Zeocin^{TM}, the number of survivors declined to 13 while only
301 five colonies withstood 2,000 μg.mL^{-1} Zeocin^{TM}. Most probably these different
302 sensitivities reflected the number of integrations of the expression cassette into *P.*
303 *pastoris*. One transformant (K1) that was resistant only to 500 μg.mL^{-1} Zeocin^{TM} was
304 selected for enzyme expression because a preliminary study indicated that it produced
305 the highest crude protein level among all transformants tested.
306
307 **Production of GH1254 in shaken flasks**
308 *Gh*1254 comprised 1026 nucleotides and encoded 341 amino acids. The SignalP
309 programme (http://www.cbs.dtu.dk/service/SignalP) predicted a signal peptide at
310 amino acids 1 to 16. Thus, the mature predicted protein consisted of 325 amino acids,
311 a molecular mass of 42 kDa and an isoelectric point of 4.6
312 (http://web.expasy.org/compute_pi/). Maximum enzyme production levels were
313 detected when the cultures were induced for 1 d using 1% (v/v) methanol. The
314 expression levels of GH1254 were low and the target proteins were only seen as faint
315 bands on SDS-PAGE (Fig.3). The target protein could not be recovered following
316 IMAC purification. Obviously shaken flasks were unsatisfactory for large-scale
317 protein production and for this reason, production of GH1254 was scaled-up in a
318 bioreactor.
319 (FIGURE 3)
320
321 **Production of GH1254 in a bioreactor**

Large-scale production of GH1254 was performed in a 7.5 L bioreactor under fed-batch conditions which lead to a maximal optical density (OD_{600}) of ~ 150 and a dry cell weight (DCW) of ~ 60 $mg.mL^{-1}$ (Fig.4).

Highest activity against MUC was detected 72 h after cultivation (35 h after induction with methanol). The optimum induction period was longer than that in shaken flasks (35 h versus 24 h) (Fig.4). This difference might be due to the different media used (BMMY versus BSM), physical conditions (temperature, pH, dissolved oxygen etc) and feed consistencies (batch versus fed-batch) (Table 1) [34].

(FIGURE 4)(TABLE 1)

Purification and Western blotting of GH1254

Crude protein yields from the yeast transformant cultures increased from 3.2 mg (or 16 $mg.L^{-1}$) in shaken flasks to 240 mg (or 80 $mg.L^{-1}$) in the fermenter. Higher specific activities against MUC were also observed (0.053 $U.mg^{-1}$ in flasks versus 0.145 $U.mg^{-1}$ in the bioreactor). After purification to homogeneity using IMAC, western-blotting methods confirmed the presence of GH1254 by using anti-His antibody (Fig. 5B) (Table 2). Purified GH1254 had a molecular mass of ~ 42 kDa (Fig. 5A). The specific activity towards MUC decreased following IMAC purification (0.5 $U.mg^{-1}$ versus 1.6 $U.mg^{-1}$) (Table 2). The reason for this is unclear; however, the higher level of MU-cellobioside hydrolysis shown by the partially purified proteins extracted from the transformant carrying pPICZαC_Gh1254 was not due to the *P. pastoris* host (without pPICZαC_Gh1254) enzymes as host crude protein extracts gave negligible MU-cellobioside activity.

(FIGURE 5)(TABLE 2)

Optimum catalytic conditions for GH1254

Optimum catalysis by GH1254 occurred at 35 °C and pH 4 (Fig. 6A, Fig. 6B). Full activity was retained after 30 min pre-incubation at 25, 30 and 35 °C, about 70% at 40 °C and 60% at 50°C (Fig.6C). The enzyme appeared to be stable for 30 min from pH 4 to pH 7, with some activity loss at pH 3 and pH 8 (Fig.6D). Interestingly, while GH1254 displayed almost undetectable catalytic activity at 45 ^{o}C (Fig. 6A), it displayed >60% residual activity upon 30 min pre-incubation at 45 ^{o}C or even at 50 ^{o}C (Fig. 6C). We have no firm explanation for this phenomenon but GH1254 most likely possesses an extraordinary capacity to refold its structure rapidly upon cooling to the assay temperature (35 ^{o}C). A similar feature was also reported in the cold-active salmon goose-type lysozyme where the recombinant enzyme showed no detectable activity at 60 ^{o}C; however, 30% activity was retained after heating the enzyme 3 h at 90 ^{o}C [35]. The enzyme was stable across all pHs tested (3 to 8) (Fig. 6D). The optimum temperature of GH1254 (35 °C) is substantially lower than cellulases from other termites including the endoglucanase from *Nasutitermes takasagoensis* (65 °C) [36], the endoglucanase from *Coptotermes formosanus* (70 °C) [37] and the β-glucosidase from *Neotermes koshunensis* (45 °C) [38].

Interestingly, a cellulase from *R. flavipes* [16] which is homologous to GH1254 (40% identical at the amino acid level) also had a low optimum temperature (30 ^{o}C). Reticulitermes are the most widespread termite pests of wood in Europe, Japan and North America [39]. Whether cellulases that are optimally active at ambient temperatures give an added advantage to termites as efficient wood or living tree decomposers remains to be determined.

Different optimum pH values have been reported for various termite cellulases. Those from *Macrotermes mülleri* displayed highest activity at pH 4.4, while a *C.*

372 *formosanus* endoglucanase showed maximum activity at pH 5.8 to 6.0 [37,40]. The
373 optimum pH for β-glucosidase from *Nasutitermes exitiosus* ranged from 2.0 to 2.8 in
374 the foregut, 2.8 to 3.8 in the rectum, and 6.8 to 7.5 in the midgut/mixed segment [41].
375 These diversities observed of termite cellulases suggest that they are independent of
376 the species but depend on the gut microbiome which constantly adapts to its
377 environment [4].
378 (FIGURE 6)
379

380 **Substrate specificities of GH1254**

381 Although MU-cellobioside (MUC) was used as the standard substrate for assaying
382 GH1254 activity, other compounds were also tested. GH1254 actively degraded *p*NP-
383 cellobioside (*p*NPC) and *p*NP-lactoside (*p*NPL) but not Avicel®, carboxymethyl
384 cellulose (CMC) or Sigmacell® cellulose (Table 3). The ability to degrade CMC is
385 regarded as an indicator of endoglucanase activity [42] while activity towards Avicel®
386 and Sigmacell® cellulose is used to detect cellobiohydrolase activity [43]. As GH
387 1254 was nonhydrolytic towards both Avicel and CMC, its identity remains elusive
388 since GHF7 cellulases include at least both cellobiohydrolases and endoglucanses
389 [10].
390 (TABLE 3)
391

392 **Kinetics of GH1254**

393 Using MUC as the substrate, the Michaelis-Menten constant (K_m) and maximum
394 velocity (V_{max}) of purified GH1254 were 1.5 mM and 1.19 μmol.min^{-1}.mg^{-1},
395 respectively (Fig.7). The substrate affinity of GH1254 towards MUC was lower than
396 that of the *T. reesei* Cel7A (towards MU-lactoside, K_m=0.70 mM) [44] and EG1

(towards MUC, K_m=0.125 mM) [45] expressed in *P. pastoris*. The maximum velocity (V_{max}) of GH1254 towards MUC (1.19 Umg^{-1}) was however higher than that of *T. reesei* CBHI (0.008 Umg^{-1} towards *p*NP-lactoside) [46]. It is critical to note that the maximum concentration of MUC being used in this study was 2.0 mM due to the low solubility of MUC at higher concentrations. Therefore, extrapolation of both K_m and V_{max} values at non-saturation substrate concentrations may result in biased kinetic readings. The turnover number (k_{cat}) value was calculated to be 0.9 s^{-1}.

(FIGURE 7)

Effect of metal ions and denaturising agents

GH1254 was incubated with metal ions and assayed for cellulase activity against MUC (Fig.8). All ions except Mg^{2+} inhibited GH1254 activity by 10 to 95% (Fe^{3+}). At 10 mM, Mg^{2+} activated GH1254 by ~ 10%. In general, the inhibitory effects of positive ions could be avoided by adding chelating agents such as ethylenediaminetetraacetic acid (EDTA) into the reaction mix [18].

Industrial applications of enzymes often involve harsh conditions and the use of denaturing agents. SDS is often used in industrial applications of enzymes but at concentrations ranging from 0.1% to 1.0% inhibited enzyme activity by ~ 35% and ~ 25%, respectively. Perhaps GH1254 can be engineered to retain high activities in the presence of SDS.

(FIGURE 8)

In summary, large quantities of a recombinant glycoside hydrolase family 7 enzyme from *C. curvignathus* were produced in *P. pastoris* using a bioreactor. The ambient temperature optimum for GH1254 may partially explain why *C. curvignathus* possesses extraordinary digestive power on tissues of living trees. GH1254 showed

highest activity at 35 ºC and maintained 70% of its activity at 25 ºC. This suggests a potential application in food industry such as fruit juice extraction and clarification [47]. This study also showed that the *P. pastoris* X-33 expression system can produce functional termite derived cellulases and can be used as an alternative to the insect cell expression system.

Acknowledgements

The authors would like to thank the Ministry of Science, Technology and Innovation (MOSTI) of Malaysia for providing the research grant 02-05-20-SF11118 and Shaman M. Gaspar (Infors South East Asia/Bumi Sains Sdn. Bhd.) for his help with the bioreactor.

References

1. Kudo, T. (2009). Termite-microbe symbiotic system and its efficient degradation of lignocellulose. *Bioscience, Biotechnololgy and Biochemistry*, *73*, 2561–2567.
2. Ohkuma, M. (2003). Termite symbiotic systems: efficient bio-recycling of lignocellulose. *Applied Microbiology and Biotechnology*, *61*:1–9.
3. US DOE. (2007) Biofuels: Bringing Biological Solutions to Energy Challenges, US Department of Energy Office of Science. http://genomicscience.energy.gov/pubs/Biofuels_Flyer_2007-2.pdf
4. King, J.H.P., Mahadi, N.M., Bong, C.F.J, Ong, K.H. & Hassan, O. (2014). Bacterial microbiome of *Coptotermes curvignathus* (Isoptera: Rhinotermitidae) reflects ,the coevolution of species and dietary pattern. *Insect Science*, *21*, 584–596.
5. Brune, A. (1998). Termite guts: The world's smallest bioreactors. *Trends in Biotechnology*, *16*,16–21.
6. Brune, A. (2014). Symbiotic digestion of lignocellulose in termite guts. *Nature Reviews*, *12*,168-180.
7. Ni, J. & Tokuda, G. (2013). Lignocellulose-degrading enzymes from termites and their symbiotic microbiota. *Biotechnology Advances*, *31*, 838–850.
8. Lo, N. & Eggleton, P. (2011). Termite phylogenetics and co-cladogenesis with symbionts, in *Biology of Termites: A Modern Synthesis* (Bignell, D.E., Roisin, Y. & Lo, N., eds), Springer Netherlands, pp. 27–50.

9. Tho, Y.P. (1992) Termites of Peninsular Malaysia in *Malayan Forest Records No. 36.* (Kirton, L.G. ed.), Forest Research Institute Malaysia, Kuala Lumpur, vol. 36 pp. 1-224.
10. Woon, J.S.K., Mackeen, M.M., Sudin, A.H., Mahadi, N.M., Ilias, R.M., Abdul Murad, A.M. & Abu Bakar, F.D. (2016) Production of an oligosaccharide-spesific cellobiohydrolase from the thermophilic fungus *Thielavia terrestris*. *Biotechnology Letters*, 38(5), 825-832.
11. Mansfield, S.D., Mooney, C. & Saddler J.N. (1999). Substrate and enzyme characteristics that limit cellulose hydrolysis. *Biotechnology Progress*, *15*, 804–816.
12. Woon, J.S.K., Mackeen, M.M., Mahadi, N.M., Ilias, R.M., Abdul Murad, A.M. & Abu Bakar, F.D. (2016). Expression and characterization of a cellobiohydrolase (CBH7B) from the thermophilic fungus *Thielavia terrestris* in *Pichia pastoris*. *Biotechnology and Applied Biochemistry*, *63*(5), 690-698.
13. Teeri, T. T. (1997). Crystalline cellulose degradation: New insight into the function of cellobiohydrolases. *Trends in Biotechnology*, *15*, 160–167.
14. Nakashima, K., Watanabe, H. & Azuma, J.I. (2002). Cellulase genes from the parabasalian symbiont *Pseudotrichonympha grassii* in the hindgut of the wood-feeding termite *Coptotermes formosanus*. *Cellular and Molecular Life Sciences*, *59*,1554–1560.
15. Tokuda, G. and Watanabe, H. (2007). Hidden cellulases in termites: revision of an old hypothesis. *Biology Letters*, *3*,336–339.
16. Sethi, A., Kovaleva, E. S., Slack, J. M., Brown, S., Buchman, G. W. & Scharf, M. E. (2013). A GHF7 cellulase from the protist symbiont community of *reticulitermes flavipes* enables more efficient lignocellulose processing by host enzymes. *Archives of Insect Biochemistry and Physiology*, *84*(4), 175–193.
17. Den Haan, R., Mcbride, J.E., Grange, D.C. La, Lynd, L.R. & Van Zyl, W.H. (2007). Functional expression of cellobiohydrolases in *Saccharomyces cerevisiae* towards one-step conversion of cellulose to ethanol. *Enzyme and Microbial Technology*, *40*, 1291–1299.
18. Wang, G., Zhang, X., Wang, L., Wang, K., Peng, F. & Wang, L. (2012). The activity and kinetic properties of cellulases in substrates containing metal ions and acid radicals. *Advances in Biological Chemistry*, *2*(11), 390–395.
19. Cregg, J.M., Vedvick, T.S. & Raschke, W.C. (1993). Recent Advances in the Expression of Foreign Genes in *Pichia pastoris*. *Bio-Technology*, *11*, 905–910.
20. Valencia, J.A, Wang, H. & Siegfried, B.D. (2014). Expression and characterization of a recombinant endoglucanase from western corn rootworm, in *Pichia pastoris*. *Journal of Insect Science*, *1*(14), 242.
21. Sambrook, J.W. & Russell, D. (2001). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harb Lab Press Cold Spring Harb, NY.
22. Schulz, M.H., Zerbino, D.R., Vingron, M. & Birney, E. (2012). Oases: robust de novo RNA-seq assembly across the dynamic range of expression levels. *Bioinformatics*, *28*(8), 1086-1092.

23. Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J. , Zhang, Z. Miller, W. & Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, *25*(17), 3389–3402.
24. Gilkes, N.R., Henrissat, B., Kilburn, D.G., Miller, R.C. Jr. & Warren, R.A. (1991). Domains in microbial beta-1, 4-glycanases: sequence conservation, function, and enzyme families. *Microbiological Reviews*, *55*(2):303-315.
25. Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, *30*, 2725–2729.
26. Lõoke, M., Kristjuhan, K. & Kristjuhan, A. (2011). Extraction of Genomic DNA from Yeast for PCR-based Applications. *Biotechniques*, *50*:325–328.
27. Wan Seman, W.M.K., Bakar, S.A., Bukhari, N.A., Gaspar, S.M., Othman, R., Nathan, S., Mahadi, N.M., Jahim, J., Murad, A.M.A. & Abu Bakar, F.D. (2014). High level expression of *Glomerella cingulata* cutinase in dense cultures of *Pichia pastoris* grown under fed-batch conditions. *Journal of Biotechnology*, *184*, 219-228.
28. Bradford, M. (1976). Rapid and Sensitive Method for Quantification of Microgram Quantities of Protein utilizing principle of Protein-Dye-Binding. *Analytical Biochemistry, 72*, 248–254.
29. Miller, G.L. (1959). Use of dinitrosalicyclic reagent for determination of reducing sugar. *Analytical Chemistry*, *31*:426– 428.
30. Lineweaver, H. & Burk, D. (1934). The determination of enzyme dissociation constants. *Journal of the American Chemical Society*, *56*(3), 658–666.
31. Hoshino, E., Shiroishi, M., Amano, Y., Nomura, M. & Kanda, T. (1997). Synergistic actions of exo-type cellulases in the hydrolysis of cellulose with different crystallinities. *Journal of Fermentation and Bioengineering*, *84*, 300–306.
32. Demain, A.L. & Vaishnav, P. (2009). Production of recombinant proteins by microbes and higher organisms. *Biotechnology Advances*, *27*(3), 297-306.
33. Tomme, P. & Claeyssens, M. (1989). Identification of a functionally important carboxyl group in cellobiohydrolase I from *Trichoderma reesei*: A chemical modification study. *FEBS Letter*, *243*(2), 239-243.
34. Humphrey, A. (1998). Shake flask to fermentor: What have we learned? *Biotechnology Progress*, *14*(1), 3–7.
35. Kyomuhendo, P., Myrnes, B. & Nilsen, I. W. (2007). A cold-active salmon goose-type lysozyme with high heat tolerance. *Cellular and Molecular Life Sciences*, *64*, 2841-2847.
36. Tokuda, G., Watanabe, H., Matsumoto, T. & Noda, H. (1997). Cellulose digestion in the wood-eating higher termite, *Nasutitermes takasagoensis* (Shiraki): distribution of cellulases and properties of endo-beta-1,4-glucanase. *Zoological Science*, *14*(1), 83-97.
37. Inoue, T., Moriya, S., Ohkuma, M. & Kudo, T. (2005). Molecular cloning and characterization of a cellulase gene from a symbiotic protist of the lower termite, *Coptotermes formosanus*. *Gene*, *349*, 67–75.

38. Ni, J., Tokuda, G., Takehara, M. & Watanabe, H. (2007). Heterologous expression and enzymatic characterization of β-glucosidase from the drywood-eating termite, *Neotermes koshunensis*. *Applied Entomology and Zoology, 42*(3), 457–463.
39. Evans, T.A., Forschler, B.T. & Grace, J.K. (2013). Biology of invasive termites: a worldwide review. *Annual review of entomology*, *58*, 455–474.
40. Rouland, C., Lenoir-Rousseaux, J.J., Mora, P. & Renoux, J. (1989). Origin of the exocellulase and the beta-glucosidase purified from the digestive tract of the fungus-growing termite *Macrotermes muelleri*. *Sociobiology*, *15*(1989), 237-246.
41. McEwen, S.E., Slaytor, M. & O'Brien, R.W. (1980). Cellobiase activity in three species of Australian termites. *Insect Biochemistry*, *10*(5), 563–567.
42. Ma, R.J., Wang, C. Y., Liu, Y.W., Sivakumar, T.R., Ren, Z.X., Fang, Y., Jia, J.Q. et al. (2014). Identification and characterization of a novel endoglucanase (CMCase) isolated from the larval gut of *Bombyx mori*. *Journal of Asia-Pacific Entomology*, *17*(1), 67–71.
43. Beukes, N. & Pletschke, B.I. (2006). Effect of sulfur-containing compounds on Bacillus cellulosome-associated "CMCase" and "Avicelase" activities. *FEMS Microbiology Letters, 264*(2), 226–231.
44. Boer, H., Teeri, T.T. & Koivula, A. (2000). Characterization of *Trichoderma reesei* cellobiohydrolase Cel7a secreted from *Pichia pastoris* using two different promoters. *Biotechnology and Bioengineering*, *69*, 486-494.
45. Akcapinar, G.B., Venturini, A., Martelli, P.L., Casadio, R. & Sezerman, U.O. (2015). Modulating the thermostability of Endoglucanase I from *Trichoderma reesei*using computational approaches. *Protein Engineering, Design and Selection*, *28*(5), 127-135.
46. Godbole, S., Decker, S.R., Nieves, R.A., Adney, W.S., Vinzant, T.B., Baker, J.O., Thomas, S.R. & Himmel, M.E. (1999). Cloning and expression of *Trichoderma reesei* cellobiohydrolase I in *Pichia pastoris*. *Biotechnological Progress*, *15*(5), 828 - 833.
47. Soares, J.F., Dal Prá, V., Kempka, A.P., Prestes, R.C., Tres, M.V., Kuhn, R.C. & Mazutti, M.A. (2016). Cellulases for Food Applications, in New and Future Developments in *Microbial Biotechnology and Bioengineering: Microbial Cellulase System Properties and Applications* (Gupta, V. ed), Elsevier, Amsterdam, pp. 201-208.

588 **Table titles**

589

590 **Table 1** Different conditions employed in the production of GH1254

591 **Table 2** Production and purification of GH7 enzyme GH1254 expressed in *Pichia*

592 *pastoris*

593 **Table 3** Substrate specificities of the *C. curvignathus* GH1254

594

595

596

597

598

599

600

601

602

603

604

605

606

607

608

609

610

611

612

613

614

615 **Table 1** Different conditions employed in the production of GH1254
616

Type of vessel	**Shaken flasks**	**Bioreactor**
Culture Media	BMGY[a] and BMMY[b]	BSM[c]
Volume	0.4 L BMGY transferred to 0.2 L BMMY for induction	3 L
Incubation temperature	30 °C	28 °C during growth phase; 25 °C during induction phase
Feed flow	Glycerol batch 0.5% (v/v) followed by methanol batch 1.0 (v/v)	Glycerol batch 2.0% (v/v) followed by glycerol fed-batch (0.125 mL.min^{-1}) and methanol fed-batch (0.125 mL.min^{-1})
Aeration rate	Uncontrolled	2.5 vvm[d]
Dissolved oxygen levels	Uncontrolled	Dissolved oxygen levels maintained at 40%
pH of the culture media	Initial pH: ~ pH 6	Maintained at pH 5

617 [a]BMGY= buffered glycerol complex medium
618 [b]BMMY= buffered methanol complex medium
619 [c]BSM = basal salts medium; 2.7 L of BSM was supplemented with 0.3 L of *Pichia* Trace Metal 1
620 (PTM1)
621 [d]vvm= volume of air per volume of medium per minute
622

623

624

625

626

627

628

629

630

631

632

633

634

Table 2 Production and purification of GHF7 enzyme GH1254 expressed in *Pichia pastoris*

	Purification steps	Volume (mL)	Activity ($U.mL^{-1}$)	Total activity (U)[a]	Total protein (mg)	Specific activity ($U.mg^{-1}$)	Purification factor[b]	Yield (%)
Shaken flaks	Crude protein	200	0.001	0.165	3.2	0.053	1	100
	Ultrafiltration	4	0.029	0.114	0.8	0.143	2.8	69.1
Bioreactor	Crude protein	3,000	0.012	34.8	240	0.145	1	100
	Cross-flow + Ultrafiltration	12	1.46	17.6	10.8	1.63	11.2	50.4
	IMAC	3	0.48	1.45	2.9	0.501	3.5	6.2

[a]U= One unit of enzyme activity was defined as the amount of product (4-methylumbelliferrone, *p*NP or reducing sugar) in μmol produced per minute under standard condition, i.e. $\mu mol.min^{-1}$.

[b]Purification factor = Ratio between specific activities of the purified samples to specific activities of the crude proteins.

[c]Yield = Percentage of total activity of the purified samples over the total activity of the crude proteins.

663 **Table 3** Substrate specificities of the *C. curvignathus* GH1254

Substrate	**MUC**	***p*NPC**	***p*NPG3**	***p*NPL**	**CMC**	**Avicel®**	**Sigmacell® cellulose**
Specific activity ($U.mg^{-1}$)	0.51	0.042	N.D[a]	0.006	N.D[b]	N.D[b]	N.D[b]

664 N.D: not detected within the concentration range of the standard chemical compounds used in the
665 calibration curves.
666 [a] The range of *p*NP concentrations used in the calibration curve was 0.03-0.21 μmol.
667 [b] The range of glucose concentrations used in the DNS calibration curve was 0.25-8.0 μmol.
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687

688 **Figure legends**

689

690 **Figure 1**. Phylogenetic comparisons of termite (GH1254, KC751534, KC751535,
691 KC751536) and selected fungal GH sequences. The tree was constructed by the
692 neighbour-joining method. The bar indicates an evolutionary distance of 0.2 %.
693 Bootstrap values (%) are indicated at the nodes. The GenBank accession numbers of
694 the GHF7 and GHF6 sequences used are as followed: *Humicola grisea* var.
695 *thermoidea* (AAD31545), *Myceliophthora heterothallica* (AEO55787) *Talaromyces*
696 *emersonii* (AF439935), *Talaromyces emersonii* (AF439936), *Thermoascus*
697 *aurantiacus* (AF478686), *Ceratocystis virescens* (AGV05124), *Trechispora*
698 *microspora* (AGV05127), *Clostridium thermophilum* (AY861347), *Clostridium*
699 *thermophilum* (AY861348), *Humicola grisea* var. *thermoidea* (BAA09785),
700 *Humicola insolens* (BAB39154), *Melanocarpus albomyces* (CAD56667),
701 *Coptotermes curvignathus* (GH1254), *Ruminococcus* spp. (CDE11952), *Clostridium*
702 *thermophilum* (DQ085790), *Reticulitermes flavipes* (KC751534, KC751535,
703 KC751536).

704

705 **Figure 2** Multiple sequence alignment and secondary protein structure prediction of
706 GH1254 from *Coptotermes curvignathus*, GHF7 enzyme from *Reticulitermes flavipes*
707 (KC751534), CBHI from *Trichoderma reesei* (P62694) and EGI from *Trichoderma*
708 *reesei* (AAA34212) generated by the PRALINE Server
709 (http://www.ibi.vu.nl/programs/pralinewww/).

710

711 **Figure 3** SDS-PAGE analysis of culture supernatants from shake flasks after
712 induction for 24 h with 1% methanol. Lane M: Protein marker "Multicolor Broad

Range" (Thermo Fisher Scientific, IL, USA); Lane 1: Crude protein from untransformed *P. pastoris* X-33; Lane 2: Crude protein from a GH1254 transformant (K1). The black arrow indicates the target protein.

Figure 4 Dry cell weight, optical density and MU-cellobioside activity during cultivation in a bioreactor of the *Pichia pastoris* GH1254 transformant (K1) at 28 °C. DCW: dry cell weight; OD_{600}: optical density of the cells.

Figure 5 A) SDS-PAGE analysis of samples from expression and purification of GH1254. Lane 1: Crude protein from untransformed *P. pastoris* X-33 cultured in shaken flasks; Lane M: Protein marker "Multicolor Broad Range" (Thermo Fisher Scientific, IL, USA); Lane 2: Crude GH1254 expressed in a bioreactor; Lane 3: Concentrated (cross-flow) crude GH1254 expressed in a bioreactor; Lane 4: Concentrated (cross-flow plus ultrafiltration using Vivaspin 10 kDa MWCO) crude GH1254 expressed in a bioreactor; Lane 5: GH1254 purified via immobilized metal-ion chromatography (IMAC). The black arrow indicates the target protein.
B) Western blotting analyses of purified GH1254. Lane M: Unstained protein marker (New England Biolabs, MA, USA); Lane 1: Negative control (crude protein from untransformed *P. pastoris* X-33); Lane 2: Purified GH1254.

Figure 6 Temperature and pH dependence of activity and stability of purified GH1254. A) Relative activity on MUC at indicated temperatures and B) at different pHs. Panels C) and D) show residual activity after 30 min preincubation at indicated temperatures (C) and pHs (D), measured with MUC at pH 4.0 and 35 °C. Relative

738 activity is calculated as the percentage of the highest specific activity in each series,
739 which were 0.53, 0.48, 0.52 and 0.47 Umg^{-1}, respectively.
740
741 **Figure 7** Lineweaver-Burk Plot of GH1254 depicting its Michaelis-Menten constant
742 (K_m) and maximum velocity (V_{max}) towards 4-methylumbelliferyl-cellobioside (MUC)
743 for 20 min assayed at 35 ^oC and pH 4.
744
745 **Figure 8** Effect of metal ions and SDS on the activity of purified GH1254. Relative
746 activities were calculated using the specific activity of the control (assayed without
747 metal ions) as the reference, taken as 100%. Asterisks (*) indicate significant
748 differences ($P<0.05$) in relative activity of a specific sample in comparison to the
749 relative activity of the control sample.
750
751
752